The EMBO Journal vol.15 no.23 pp.6766–6774, 1996

The G222D mutation in elongation factor Tu inhibits the codon-induced conformational changes leading to GTPase activation on the ribosome

Erik Vorstenbosch, Tillmann Pape[1], Marina V.Rodnina[1], Barend Kraal and Wolfgang Wintermeyer[1,2]

Leiden Institute of Chemistry, Department of Biochemistry, Leiden University, 2300 RA Leiden, The Netherlands and [1]Institute of Molecular Biology, University of Witten/Herdecke, 58448 Witten, Germany

[2]Corresponding author

Elongation factor Tu (EF-Tu) from *Escherichia coli* carrying the mutation G222D is unable to hydrolyze GTP on the ribosome and to sustain polypeptide synthesis at near physiological Mg^{2+} concentration, although the interactions with guanine nucleotides and aminoacyl-tRNA are not changed significantly. GTPase and polypeptide synthesis activities are restored by increasing the Mg^{2+} concentration. Here we report a pre-steady-state kinetic study of the binding of the ternary complexes of wild-type and mutant EF-Tu with Phe-tRNA^{Phe} and GTP to the A site of poly(U)-programed ribosomes. The kinetic parameters of initial binding to the ribosome and subsequent codon–anticodon interaction are similar for mutant and wild-type EF-Tu, independent of the Mg^{2+} concentration, suggesting that the initial interaction with the ribosome is not affected by the mutation. Codon recognition following initial binding is also not affected by the mutation. The main effect of the G222D mutation is the inhibition, at low Mg^{2+} concentration, of codon-induced structural transitions of the tRNA and, in particular, their transmission to EF-Tu that precedes GTP hydrolysis and the subsequent steps of A-site binding. Increasing the Mg^{2+} concentration to 10 mM restores the complete reaction sequence of A-site binding at close to wild-type rates. The inhibition of the structural transitions is probably due to the interference of the negative charge introduced by the mutation with negative charges either of the 3′ terminus of the tRNA, bound in the vicinity of the mutated amino acid in domain 2 of EF-Tu, or of the ribosome. Increasing the Mg^{2+} concentration appears to overcome the inhibition by screening the negative charges.

Keywords: codon recognition/fluorescence stopped-flow/ mechanism of A-site binding/pre-steady-state kinetics/ protein synthesis

Introduction

The function of EF-Tu in protein biosynthesis is to promote the binding of the correct aminoacyl-tRNA to the ribosome in response to the codon present in the A site. EF-Tu·GTP forms a high affinity complex with aminoacyl-tRNA ('ternary complex') which enters the ribosomal A site rapidly. The binding proceeds in several steps. After the initial contact of the ternary complex with the ribosome, codon recognition takes place, which is followed by the hydrolysis of GTP, the dissociation of the factor and the accommodation of aminoacyl-tRNA in the A site. The molecular details of how the GTPase activity of EF-Tu is triggered during the interaction with elongating ribosomes are not known. Time-resolved fluorescence studies have shown that, in response to codon–anticodon recognition, the ternary complex undergoes a series of conformational changes that involve both tRNA and EF-Tu and result in a 10^4-fold enhancement of the GTPase activity (Rodnina *et al.*, 1994, 1995, 1996). These results suggest that the tRNA that has recognized the cognate codon in the 30S decoding center is acting as a GTPase-activating effector of EF-Tu. Presumably, the ribosome is also involved by providing binding interactions that promote the formation of the activated structure of EF-Tu (see Discussion).

The crystal structure of the GTP form of EF-Tu from thermophilic bacteria (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993) revealed strong similarities between the G domain of EF-Tu and p21 (Pai *et al.*, 1990). In line with this structural similarity, the region encompassing amino acids 40–60 of domain 1 (effector region) of EF-Tu was proposed to be a site for ribosome interaction (Clark *et al.*, 1990). There is evidence from proteolysis experiments supporting the view that the region formed by residues 52–60 serves the function of an effector binding region (Peter *et al.*, 1990), i.e. responds to an external interaction by a conformational change which, in turn, triggers GTP hydrolysis. While these data emphasize the importance of the effector region of EF-Tu for GTPase activation, the direct involvement of the ribosome in operating the GTPase switch remains questionable. According to the crystal structure of the ternary complex EF-Tu·GDPNP·Phe-tRNA^{Phe} (Nissen *et al.*, 1995), the effector region is in direct contact with the aminoacyl-tRNA molecule, along with residues located in domain 2 (*Escherichia coli* EF-Tu residues 218–225, 260–265 and 288–291) and domain 3 (*E.coli* residues 318–319, 329–338, 363 and 379).

Domain 2 of EF-Tu has been implicated in the interaction with the ribosome, since mutations in this region, such as G222D and G280A, affect the ribosome-dependent functions of EF-Tu (Swart *et al.*, 1987; Tubulekas and Hughes, 1993). EF-Tu(G222D) does not promote polypeptide synthesis at Mg^{2+} concentrations below 7 mM (Swart *et al.*, 1987), although the interactions with guanine nucleotides and aminoacyl-tRNA are not changed significantly (Duisterwinkel *et al.*, 1984; Swart *et al.*, 1987; Abrahams *et al.*, 1990). At low Mg^{2+} concentrations, the interaction of the ternary complex of EF-Tu(G222D)·GTP and aminoacyl-tRNA with the ribosome does not lead to GTP hydrolysis and subsequent peptide bond formation;

elevated Mg^{2+} levels are needed to restore these reactions (Swart *et al.*, 1987). EF-Tu(G222D) is sensitive to kirromycin *in vitro* but is not blocked on the ribosome in the presence of the antibiotic (Duisterwinkel *et al.*, 1981; Van der Meide *et al.*, 1981). It was found originally in a kirromycin-resistant *E.coli* strain together with the kirromycin-resistant EF-Tu(A375T). Though unable to sustain *in vivo* protein synthesis by itself, EF-Tu(G222D) can be complemented by EF-Tu(A375T), and the resulting phenotype is remarkable for its enhanced translational readthrough and frameshifting (Vijgenboom *et al.*, 1985; Vijgenboom and Bosch, 1989).

In the present pre-steady-state kinetic study, the ternary complexes of EF-Tu(G222D) or wild-type EF-Tu with GTP and Phe-tRNA^{Phe} are compared in binding to the A site of poly(U)-programed ribosomes at 5 and 10 mM Mg^{2+}. EF-Tu(G222D) was modified with a C-terminal His tag for efficient purification by Ni^{2+} affinity chromatography. Wild-type EF-Tu was used both with and without the His tag (Boon *et al.*, 1992): no significant differences in kinetic parameters were observed. We have characterized the codon-independent initial binding and the resulting complex by time-resolved (stopped-flow) and steady-state fluorescence measurements using a fluorescent tRNA derivative, tRNA^{Phe}(Prf16/17). The subsequent codon-dependent steps of A-site binding were studied by the fluorescence stopped-flow technique using fluorescent tRNA^{Phe}(Prf16/17) (Rodnina *et al.*, 1994), carrying covalently bound proflavin replacing dihydrouracil 16 or 17 in the D loop, and a fluorescent GTP derivative, 3′-*O*-(*N*-methylanthranilyl)-2′-deoxyguanosine triphosphate (mant-dGTP, Rodnina *et al.*, 1995). The rates of GTP hydrolysis and peptide bond formation were measured by the quench-flow technique. At 10 mM Mg^{2+}, the kinetics of A-site binding is practically the same for the ternary complexes containing wild-type or mutant EF-Tu. At 5 mM Mg^{2+}, the kinetics of initial ribosome binding and codon recognition is still identical for wild-type and mutant EF-Tu; however, the subsequent conformational transitions leading to GTP hydrolysis, accommodation of aminoacyl-tRNA to the A site and peptide bond formation are abolished with EF-Tu(G222D). The implications of the present observations for the mechanism of transmission of the conformational signal created by codon–anticodon interaction with EF-Tu and for translational control are discussed.

Results

Initial ribosome binding of EF-Tu(G222D)·GTP·aminoacyl-tRNA

To study the initial binding of the mutant ternary complex to the ribosome, the interaction of EF-Tu(G222D)·GTP·Phe-tRNA^{Phe}(Prf16/17) with poly(A)-programed ribosomes was monitored by fluorescence. To control that the observed effects are not due to the presence of the His tag on the mutant protein, the experiments were run in parallel with wild-type EF-Tu containing the His tag. For all experiments, the respective ternary complex was purified by gel filtration from deacylated tRNA and excess EF-Tu. The stability of the ternary complexes containing EF-Tu(G222D), EF-Tu(wt-His) or wild-type EF-Tu was similar. The isolated ternary complex was mixed rapidly with the pre-formed ribosome complex in a stopped-flow apparatus and the fluorescence signal monitored.

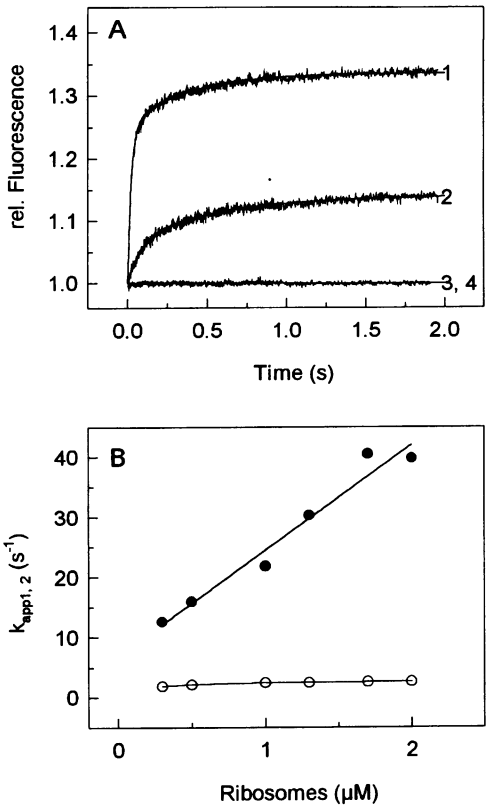

Fig. 1. Effect of G222D mutation on the kinetics of initial ribosome binding of the ternary complex. (**A**) Time course of binding of wild-type EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) (1) and EF-Tu(G222D)·GTP·Phe-tRNA^{Phe}(Prf16/17) (2) to poly(A)-programed ribosomes at 10 mM Mg^{2+} followed by proflavin fluorescence; controls (3 and 4) were performed by mixing the respective ternary complex with buffer. Concentrations of the EF-Tu and ribosome complexes were 0.1 and 0.3 μM, respectively. Parameters of two-exponential fits: (1) k_{app1} = 42/s, A_1 = 25%, k_{app2} = 3/s, A_2 = 8%; (2) k_{app1} = 13/s, A_1 = 7%, k_{app2} = 2/s, A_2 = 8%. (**B**) Dependence on ribosome concentration of initial binding of ternary complex containing EF-Tu(G222D) at 10 mM Mg^{2+}, k_{app1} (●) and k_{app2} (○). Fitting on the basis of a two-step sequential model (see Materials and methods) yielded the parameters given in Table I.

The binding of the ternary complex to poly(A)-misprogramed ribosomes at 10 mM Mg^{2+} leads to a rapid, biphasic increase of the fluorescence of proflavin in the D loop (Figure 1A). In the experiment with EF-Tu(wt-His) (trace 1), apparent rate constants of 42 and 3/s were obtained, i.e. the same as with EF-Tu without His tag (Rodnina *et al.*, 1996). For EF-Tu(G222D) (trace 2), the respective rate constants were 13 and 2/s.

With increasing ribosome concentration, k_{app1} increases linearly, while k_{app2} saturates (Figure 1B). This suggests a two-step mechanism where the second-order binding step is followed by a first-order rearrangement (Bernasconi, 1976). The origin of the second step is not clear at present; it probably reflects a local movement of the fluorophor which is not of immediate relevance for the mechanism of A-site binding. On the basis of a sequential two-step model, the rate constants for 10 mM Mg^{2+} were obtained from the titration curves (Table I); the rate constants for 5 mM Mg^{2+} have been obtained by numerical integration of the reaction progress curves on the basis of the

Table I. Effect of G222D mutation in EF-Tu on the kinetics of initial binding at 5 and 10 mM Mg^{2+}

Rate constants	G222D		Wild type	
	10 mM	5 mM	10 mM	5 mM
k_1 (μM^{-1} s^{-1})	19 ± 2	12 ± 1	59 ± 5	10 ± 1
k_{-1} (s^{-1})	6 ± 1	31 ± 5	24 ± 2	29 ± 2
k_2 (s^{-1})	2 ± 1	2 ± 1	4 ± 1	3 ± 1
k_{-2} (s^{-1})	1 ± 1	6 ± 1	1 ± 1	5 ± 1

known fluorescence contributions of the intermediate states (Materials and methods). For comparison, the corresponding values for wild-type EF-Tu are also given in Table I (Rodnina *et al.*, 1996).

Lowering the Mg^{2+} concentration from 10 to 5 mM decreases the forward and increases the backward rate constants of the first step. The rate constants of the second step depend only weakly on Mg^{2+} concentration for both the mutant and wild-type EF-Tu. Notably, at 10 mM Mg^{2+}, the rate constants of initial binding are somewhat lower for EF-Tu(G222D) as compared with wild-type EF-Tu, while at 5 mM Mg^{2+} they are practically identical.

The equilibrium constants, K_d, of initial binding of EF-Tu(G222D)·GTP·Phe-$tRNA^{Phe}$ (Prf16/17) to poly(A)-programed ribosomes at different Mg^{2+} concentrations were determined from fluorescence titrations. Under all conditions, saturating titration curves were obtained which were then fitted assuming one binding site for the ternary complex (Figure 2A). The affinity of the ternary complex for poly(A)-programed ribosomes increases by a factor of ~10 when the Mg^{2+} concentration is increased from 5 to 10 mM. The plot of log(K_d) versus log(Mg^{2+}) (Figure 2B) reveals that four Mg^{2+} ions are involved in initial binding, similar to the five Mg^{2+} ions found for wild-type EF-Tu (Rodnina *et al.*, 1996).

Thus, both kinetic and equilibrium parameters of initial binding to the ribosome are practically identical at 5 mM Mg^{2+} and similar at 10 mM Mg^{2+} for the ternary complexes containing wild-type and mutant EF-Tu, as is the number of Mg^{2+} ions participating in the stabilization of the initial complex. This shows that the mutation G222D does not appreciably affect the initial interaction of the ternary complex with the ribosome, and that the phenotype at low Mg^{2+} is not due to an impaired ribosome interaction. Hence, the mutant protein must be deficient in a step downstream of the formation of the initial binding complex, i.e. codon recognition or GTPase activation.

Codon–anticodon recognition is not affected by the G222D mutation

To study codon-dependent steps of A-site binding, the interaction of the EF-Tu(G222D)·GTP·Phe-$tRNA^{Phe}$ (Prf16/17) complex with poly(U)-programed ribosomes was monitored by fluorescence. As shown previously (Rodnina *et al.*, 1994), in the case of wild-type ternary complex, codon recognition leads to a biphasic change of the proflavin fluorescence (Figure 3A, trace 1). The two steps reflect the transient formation of a conformational state of the tRNA in the ternary complex that is induced by codon–anticodon recognition (fluorescence increase) and then (after GTP hydrolysis, see below) rearranges

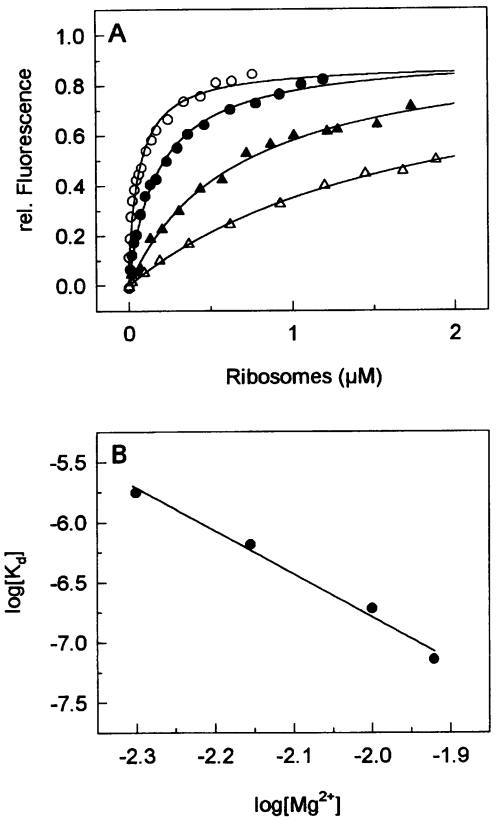

Fig. 2. Mg^{2+} dependence of initial ribosome binding of ternary complex containing EF-Tu(G222D). (**A**) Fluorescence titration of EF-Tu(G222D)·GTP·Phe-$tRNA^{Phe}$(Prf16/17) with poly(A)-programed ribosomes at 5 (△), 7 (▲), 10 (●) and 12 (○) mM Mg^{2+}. (**B**) Mg^{2+} dependence of K_d values determined from the titrations shown in (A).

further to the final A site-bound state of the tRNA (fluorescence decrease) (Rodnina *et al.*, 1994).

A qualitatively different picture is observed with mutant EF-Tu (Figure 3A, trace 2). While the same final fluorescence level is reached eventually, the high fluorescence of the intermediate formed with wild-type EF-Tu is not observed with the mutant. The rate of the first step, which reflects codon–anticodon recognition, is similar to that observed with wild-type EF-Tu (52/s versus 61/s at 2 μM ribosome concentration; Table II); however, the amplitude is lowered ~2.5-fold. The rate of the second step is reduced somewhat with the mutant ternary complex (4/s versus 9/s for the wild type). The different signal changes suggest that the codon-induced conformational rearrangement of aminoacyl-tRNA in the mutant ternary complex is different from that in the wild-type complex. As a consequence of the smaller initial amplitude, the rearrangement of the intermediate to the final state is accompanied by a further fluorescence increase, rather than a decrease, to reach the same final level as seen with wild-type EF-Tu. The slower reaction characterizes the accommodation of aminoacyl-tRNA in the A site after GTP hydrolysis (Rodnina *et al.*, 1994). The finding of the same fluorescence in the final state then suggests that the A-site-bound state of the aminoacyl-tRNA is not affected by the mutation in EF-Tu.

To compare the rate constants of codon–anticodon recognition in the mutant and wild-type ternary complexes, the concentration dependence was studied. k_{app1} increases with increasing ribosome concentration and approaches

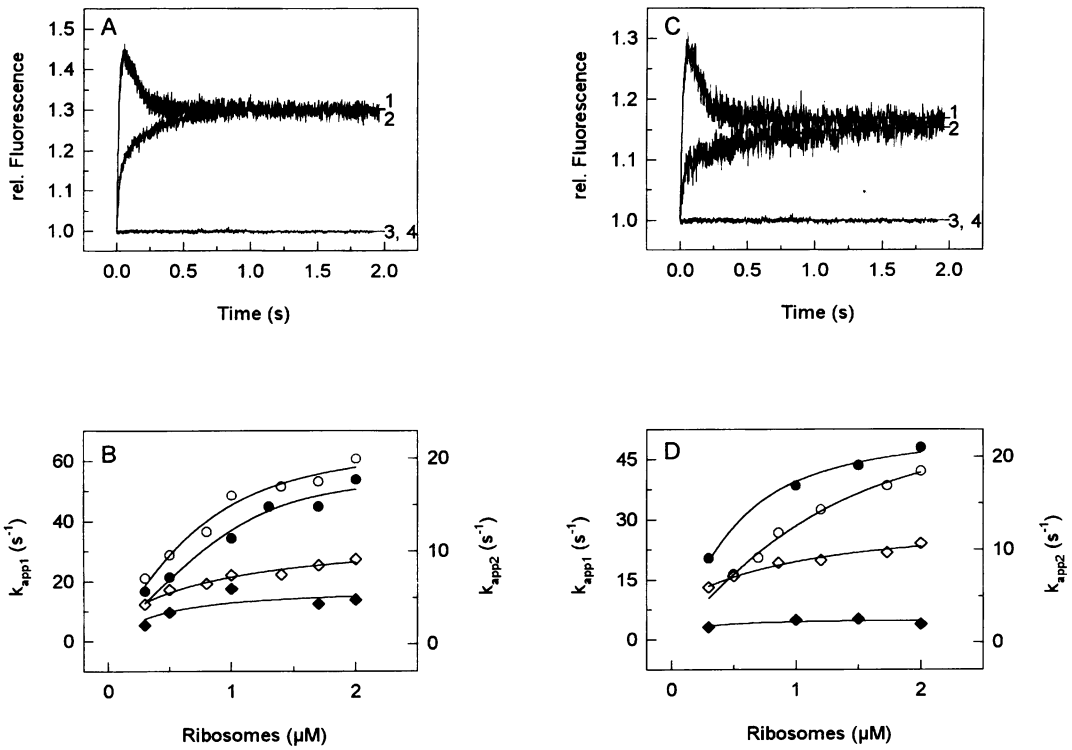

Fig. 3. Effect of G222D mutation on the kinetics of ternary complex binding to the A site. (**A**) Time course of binding of EF-Tu·GTP·Phe-$tRNA^{Phe}$(Prf16/17) (1) and EF-Tu(G222D)·GTP·Phe-$tRNA^{Phe}$(Prf16/17) (2) to poly(U)-programed ribosomes containing AcPhe-$tRNA^{Phe}$ in the P site at 10 mM Mg^{2+}, monitored by proflavin fluorescence; controls (3 and 4) were without ribosomes. Concentrations after mixing were 0.1 μM ternary complex and 2 μM ribosomes. Parameters of two-exponential fits: (1) k_{appl}= 61/s, A_1 = 56%, k_{app2} = 9/s, A_2 = –26%; (2) k_{appl} = 54/s, A_1 = 17%, k_{app2} = 4/s, A_2 = 13%. (**B**) Concentration dependence of k_{appl} (●, ○) and k_{app2} (◆, ◇) from (A) of wild-type (○, ◇) and mutant (●, ◆) ternary complex at 10 mM Mg^{2+}. (**C**) As in (A), except 5 mM Mg^{2+}. Parameters of two-exponential fits: (1) k_{appl} = 42/s, A_1 = 45%, k_{app2} = 11/s, A_2 = –28%; (2) k_{appl} = 48/s, A_1 = 9%, k_{app2} = 2/s, A_2 = 6%. (**D**) As (B), except 5 mM Mg^{2+}.

Table II. Kinetic steps of codon-dependent A-site binding of G222D and wild-type ternary complexes[a]

Step	G222D		Wild type	
	10 mM	5 mM	10 mM	5 mM
Codon recognition/conformational change of aa-tRNA[b]	52 ± 2	48 ± 4	61 ± 1	42 ± 1
Conformational change of EF-Tu	44 ± 5	–[c]	57 ± 2	27 ± 1
GTP hydrolysis	32 ± 6	–	55 ± 4	27 ± 2
Accommodation of aa-tRNA	4 ± 1	–	9 ± 1	11 ± 1
Dissociation of EF-Tu·GDP	4 ± 1	–	4 ± 1	6 ± 1
Peptide bond formation	4 ± 1	–	4 ± 1	5 ± 1

[a]Apparent rate constants (s^{-1}) were measured with 0.1 μM of ternary complexes and near-saturating (2 μM) ribosome concentration at 10 and 5 mM Mg^{2+}.
[b]The amplitude of fluorescence change was smaller with G222D.
[c]Not observed.

saturation at ~70 and 60/s for wild-type and mutant ternary complexes, respectively (Figure 3B). Since the backward reaction after codon binding is negligible for the cognate ternary complex (Karim and Thompson, 1986), the saturation level of k_{appl} provides a good estimate for the forward rate constant of codon–anticodon recognition. Before saturation is reached, the rate of the recognition step is limited by the preceding second-order step of initial binding to the ribosome.

Decreasing the Mg^{2+} concentration to 5 mM essentially has no influence on codon recognition by the wild-type ternary complex (Figure 3C). The high fluorescence intermediate is formed, although the lower amplitude indicates some structural difference, and the rate constant of codon–anticodon recognition reaches saturation (~50/s) only at higher ribosome concentrations (Figure 3D). In the mutant ternary complex, the fluorescence increase due to codon recognition is smaller (Figure 3C), while the rate constant of codon recognition remains similar to that of the wild-type, ~50/s. The conclusion is that, in mutant EF-Tu(G222D), the codon recognition step is not affected appreciably. Rather, the conformational changes of the ternary complex following codon recognition are blocked at the lower Mg^{2+} concentration.

The slow step, characterized by k_{app2}, at 10 mM Mg^{2+} also exhibits saturating behavior, the rate observed with the mutant (4/s) being somewhat lower than that observed with the wild-type (9/s) (Figure 3B). The difference is

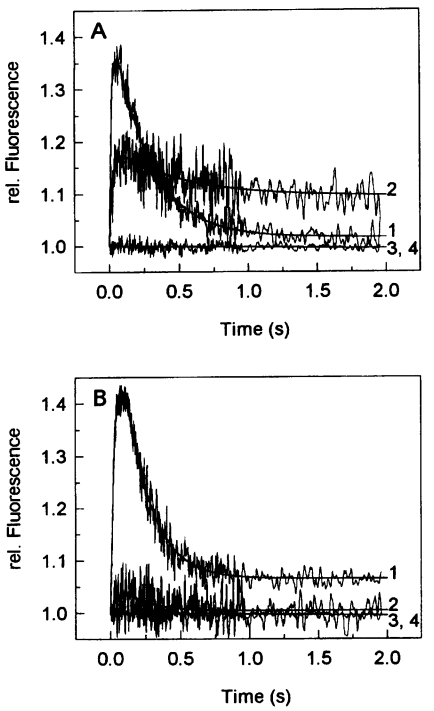

Fig. 4. Effect of G222D mutation on codon-induced rearrangement of EF-Tu. (**A**) Time course of binding of EF-Tu·mant-dGTP·Phe-tRNA^{Phe} (1) and EF-Tu(G222D)·mant-dGTP·Phe-tRNA^{Phe} (2) to poly(U)-programed ribosomes containing AcPhe-tRNA^{Phe} in the P site at 10 mM Mg^{2+}, monitored by the fluorescence of mant-dGTP; controls (3 and 4) were without ribosomes. Concentrations after mixing were 0.1 μM ternary complex and 2 μM ribosomes. Parameters of two-exponential fits: (1) k_{app1} = 57/s, A_1 = 46%, k_{app2} = 4/s, A_2 = –45%; (2) k_{app1} = 44/s, A_1 = 19%, k_{app2} = 2/s, A_2 = –10%. (**B**) As in (A), except 5 mM Mg^{2+}: (1) k_{app1} = 27/s, A_1 = 76%, k_{app2} = 6/s, A_2 = –70%.

much more pronounced at 5 mM Mg^{2+}; while the wild-type behavior is essentially not changed, the mutant shows a slow step (k_{app2} ~2/s) with small amplitude (Figure 3C) which does not show any concentration dependence (Figure 3D). A similar step was observed previously during A-site binding of non-cognate ternary complex (Rodnina *et al.*, 1996); it probably reflects a local rearrangement around the fluorophor not directly related to the process of A-site binding. We conclude that the G222D mutation in EF-Tu not only affects the conformational change following codon recognition but also the subsequent step of tRNA accommodation in the A site. The effect is probably indirect, since, as addressed in the following, the mutation, at the lower Mg^{2+} concentration, leads to an inhibition of GTP hydrolysis which, in the reaction sequence established with wild-type EF-Tu, necessarily precedes the accommodation step.

The G222D mutation inhibits a conformational transition of EF-Tu preceding GTP hydrolysis at 5 mM Mg^{2+}

To monitor conformational changes of EF-Tu during binding of the ternary complex to the A site, a fluorescent GTP derivative, mant-dGTP, was utilized. A change in mant-dGTP fluorescence was shown to reflect a codon-induced conformational rearrangement of EF-Tu preceding GTP hydrolysis (Rodnina *et al.*, 1995).

Similarly to the wild-type ternary complex, the interaction of EF-Tu(G222D)·mant-dGTP·Phe-tRNA^{Phe} with the ribosome at 10 mM Mg^{2+} results in a biphasic fluorescence change. A rapid increase in fluorescence reflects the formation of a transient intermediate which is consumed further in a slower reaction, resulting in a fluorescence decrease not far from the initial level. The rate constants of the reaction are lowered by the G222D mutation, from 57/s for the wild-type ternary complex to 44/s (Figure 4A). The amplitude of the fluorescence change is also somewhat smaller.

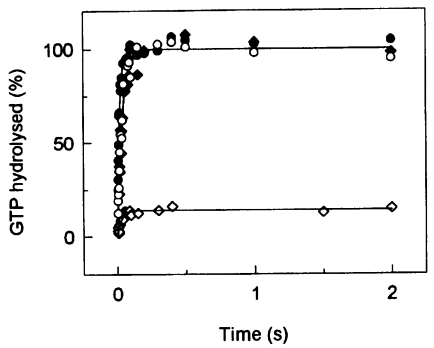

Fig. 5. Effect of G222D mutation on GTP hydrolysis by EF-Tu. The time course of GTP hydrolysis during binding of EF-Tu·[γ-^{32}P]GTP·Phe-tRNA^{Phe} (●, ○) or EF-Tu(G222D)·[γ-^{32}P]GTP·Phe-tRNA^{Phe} (◆, ◇) to poly(U)-programed ribosomes containing AcPhe-tRNA^{Phe} in the P site was measured by quench-flow at 10 mM Mg^{2+} (closed symbols) and 5 mM Mg^{2+} (open symbols). Data are plotted relative to the reaction obtained in the fully active system.

At 5 mM Mg^{2+}, the rate of the conformational rearrangement of the wild-type EF-Tu·mant-dGTP·Phe-tRNA^{Phe} is decreased to ~30/s (Figure 4B), whereas with EF-Tu(G222D)·mant-dGTP·Phe-tRNA^{Phe}, practically no further fluorescence change was observed. Since the experiment was performed at near-saturating ribosome concentration (2 μM), the loss of the fluorescence change is due to the inhibition of the conformational rearrangement, rather than to insufficient ribosome binding at the lower Mg^{2+} concentration.

Effect of the G222D mutation on GTP hydrolysis, A site accommodation of aminoacyl-tRNA and peptide bond formation

The rates of GTP hydrolysis were measured by the quench-flow technique at near-saturating ribosome concentration, i.e. the measured rates are representative of forward rate constants. At 10 mM Mg^{2+}, the rate constants of GTP hydrolysis are 55 and 32/s for wild-type and mutant EF-Tu, respectively (Figure 5). At 5 mM Mg^{2+}, the rate constant is reduced to 27/s for the wild type; practically no GTP hydrolysis is observed in the ternary complex containing mutant EF-Tu.

The release of aminoacyl-tRNA from EF-Tu to enter the A site (accommodation) and the dissociation of EF-Tu·GDP was monitored by the fluorescence of Phe-tRNA^{Phe}(Prf16/17) and mant-dGTP, respectively (Rodnina *et al.*, 1994, 1995). As seen in Figure 3, the high fluorescence intermediate rearranges into a state with lower fluorescence which is, nevertheless, somewhat higher than the initial level of free ternary complex. The rearrangement (breakdown of high fluorescence intermediate and accommodation in the A site) gives rise to a slower step observed upon binding of EF-Tu·GTP·Phe-

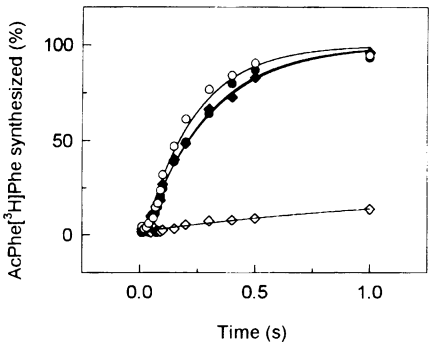

Fig. 6. Effect of G222D mutation on peptide bond formation. The time course of peptide bond formation during binding of EF-Tu·GTP·[^{3}H]Phe-tRNA^{Phe} (●, ○) and of EF-Tu(G222D)·GTP·[^{3}H]Phe-tRNA^{Phe} (◆, ◇) to poly(U)-programed ribosomes containing AcPhe-tRNA^{Phe} in the P site was measured by quench-flow at 10 mM Mg^{2+} (closed symbols) and 5 mM Mg^{2+} (open symbols). AcPhe[^{3}H]Phe was determined by HPLC analysis. Data are plotted relative to the reaction obtained in the fully active system.

tRNA^{Phe}(Prf16/17) to the A site (Figure 3A). The rearrangement and/or dissociation of EF-Tu·GDP after GTP hydrolysis is reflected in a decrease in mant-dGTP fluorescence (Figure 4; Rodnina *et al.*, 1995). To get comparable numbers, i.e. rates representing rate constants, both steps were measured at near-saturating ribosome concentration with EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) (cf. Figure 3B and D) and with EF-Tu·mant-dGTP·Phe-tRNA^{Phe} (Table II).

At 10 mM Mg^{2+}, the rate constant of accommodation of aminoacyl-tRNA in the A site is decreased only ~2-fold with the mutant compared with the wild-type EF-Tu, while the rate constants of dissociation of EF-Tu·GDP are the same (Table II). Lowering the Mg^{2+} concentration does not affect the two steps with wild-type EF-Tu. However, in the mutant ternary complex EF-Tu(G222D)·GTP·Phe-tRNA^{Phe}, both steps are absent at 5 mM Mg^{2+}.

The rates of dipeptide formation after binding of EF-Tu·GTP·Phe-tRNA^{Phe} to the A site again were measured by the quench-flow technique at near-saturating ribosome concentration. As shown in Figure 6, the rate constants of peptide bond formation are the same for wild-type and mutant EF-Tu at 10 mM Mg^{2+}. At 5 mM Mg^{2+}, the rate of dipeptide formation with wild-type ternary complex is not changed, whereas hardly any formation of dipeptides is observed with mutant EF-Tu (Figure 6).

Discussion

According to the crystal structure of the ternary complex of *Thermus aquaticus* EF-Tu, Gly222 in *E.coli* EF-Tu is located in one of the protruding loops of domain 2 (see also Figure 7). This loop (residues 218–225), which connects β strands a_2 and b_2, forms, together with the loop (residues 260–265) between the β strands d_2 and e_2, a pocket for the 3′-terminal adenine (Nissen *et al.*, 1995). The residues close to Gly222 line the pocket for the amino acid in Phe-tRNA^{Phe} (Phe218, Asp216, Glu215 and Thr228). The conserved Ile220 forms part of the hydrophobic platform on one side of the 3′-terminal adenine of the tRNA. Otherwise, Gly222 does not appear to participate directly in the interactions with aminoacyl-tRNA and contributes mainly to the conformation of the loop. Most probably, the mutation of Gly222 to Asp does not change the structure of EF-Tu overall or locally in the loop, since Asp is able to adopt the same role in the formation of this type of β-turns as Gly (Richardson and Richardson, 1990). An effect on the flexibility cannot be excluded, however.

The G222D mutation of EF-Tu does not affect interactions with GTP, GDP and kirromycin (Duisterwinkel *et al.*, 1984; Swart *et al.*, 1987). Also the binding of aminoacyl-tRNA is only marginally affected, the K_d of the EF-Tu·GTP·aminoacyl-tRNA complex being increased ~4-fold (Abrahams *et al.*, 1990) or less (E.Vorstenbosch, unpublished results). Thus the most probable explanation for the inability of the mutant to sustain protein synthesis at physiological Mg^{2+} concentrations was a defective interaction with the ribosome (Swart *et al.*, 1987).

EF-Tu-dependent binding of aminoacyl-tRNA to the A site proceeds in several steps (Rodnina *et al.*, 1994). First, the initial binding complex of EF-Tu·GTP·aminoacyl-tRNA with the ribosome is formed (Rodnina *et al.*, 1996). This complex is codon unspecific, labile and the extent of binding strongly depends on Mg^{2+} concentration. At 10 mM Mg^{2+}, the association and dissociation rate constants of initial binding of the mutant EF-Tu(G222D)·GTP·Phe-tRNA^{Phe} complex to the ribosome are only ~3-fold reduced in comparison with the wild-type, while at 5 mM Mg^{2+} they are practically equal, and both EF-Tu complexes maintain comparable equilibrium binding constants. The Mg^{2+} dependence of the affinity of the initial binding complex is also very similar for the mutant and wild-type ternary complexes. This suggests that (i) the binding of the ternary complex to the ribosome is not affected by the G222D mutation, and (ii) the rescue of EF-Tu(G222D) activity in protein synthesis by elevated Mg^{2+} is not due to improved ribosome binding of the mutant complex.

The step following initial binding is codon recognition. This step of ternary complex binding is also not affected appreciably by the G222D mutation at either 5 or 10 mM Mg^{2+}. By cognate codon–anticodon interaction, the complex is stabilized to a large extent, so that it can be isolated by nitrocellulose filtration. This is consistent with the previous finding that EF-Tu(G222D) still promotes stable binding of aminoacyl-tRNA to the ribosome (Swart *et al.*, 1987).

We have shown previously that, following the binding of the ternary complex to the ribosome, codon recognition leads to a conformational change in the aminoacyl-tRNA (anticodon and D loops) which is coupled to the formation of a transient conformational state of the G domain, monitored by increased mant-dGTP fluorescence, that immediately precedes GTP hydrolysis (Rodnina *et al.*, 1994, 1995). The formation of the intermediate state was also observed with EF-Tu(G222D) at 10 mM Mg^{2+}, although the fluorescence of mant-dGTP in the intermediate state, as of the fluorescent label in the tRNA (see above), was somewhat lower. These observations suggest that, at 10 mM Mg^{2+}, EF-Tu(G222D)·GTP·Phe-tRNA^{Phe} is able to undergo the conformational change induced by codon recognition and leading to GTP hydrolysis, although the structural details of the rearrangement seem to be somewhat different, compared with wild-type EF-Tu.

In contrast, with mutant EF-Tu, practically no change

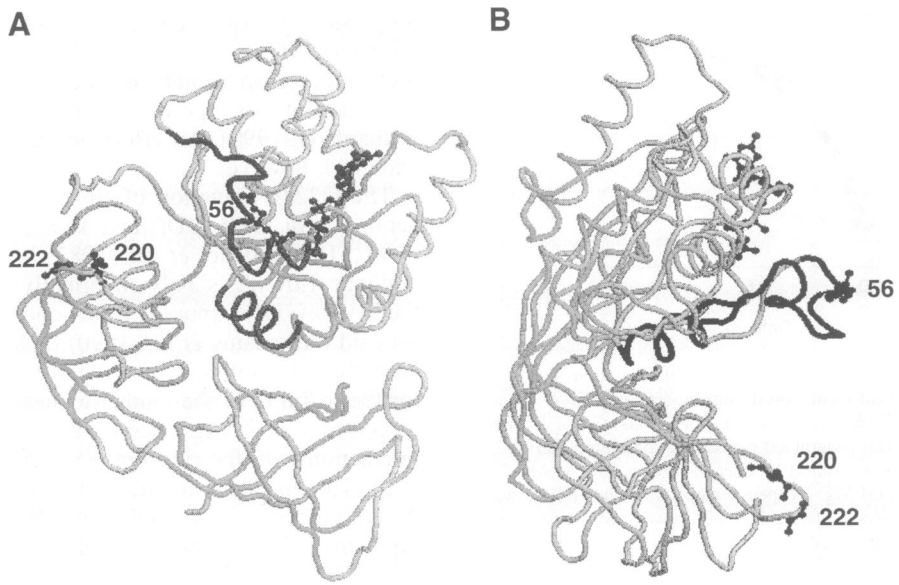

Fig. 7. Structure of *Thermus aquaticus* EF-Tu·GMPPNP (Kjeldgaard *et al.*, 1993). (**A**) Front view with domains 1 (top), 2 (bottom left) and 3 (bottom right). (**B**) Side view showing the narrow cleft between domains 1 and 2 for the accommodation of the 3′ aminoacyl acceptor arm. In domain 1, the two switch regions of the effector loop and helix B are dark and the nucleotide is drawn as a ball and stick model. Also depicted as balls are the positions of Lys56 on the effector loop (methylation attenuates GTPase activity), of Ile220 (hydrophobic contact with 3′-terminal adenine of tRNA) and of G222 (mutation to Asp blocks codon-induced signal transmission to GTPase center); all numbers are for *E.coli* EF-Tu.

of mant-dGTP fluorescence was observed at 5 mM Mg^{2+}. Since codon recognition is not affected by the mutation, this observation suggests that, at 5 mM Mg^{2+}, the transmission to EF-Tu of the signal created by codon–anticodon interaction is suppressed, as are the subsequent steps, including GTP hydrolysis, accommodation of aminoacyl-tRNA in the A site and peptide bond formation. At 10 mM Mg^{2+}, these steps are practically unaffected by the mutation; the rates of GTP hydrolysis and aminoacyl-tRNA accommodation in the A site are reduced at most 2-fold, and the rate of peptide bond formation is unchanged.

In the ternary complex, the aminoacyl acceptor arm of the tRNA has numerous contacts with the G domain, in particular with the switch regions (dark regions in Figure 7) of the effector loop and helix B (Nissen *et al.*, 1995). A close contact between the tRNA and the effector loop is also indicated by the protection by aminoacyl-tRNA binding of Lys56 (indicated in Figure 7) against proteolytic cleavage. It is also known that this residue becomes dimethylated when the cells enter the stationary phase, and it has been shown that the modification attenuates the tRNA-stimulated GTPase activity of EF-Tu (van Noort *et al.*, 1986). It is conceivable, therefore, that a codon-induced conformational change in the aminoacyl-tRNA affects these contacts and influences the conformation of the G domain of EF-Tu in such a way that the GTPase is activated. On the opposite side of the narrow cleft that forms the 3′ acceptor end binding site, the protruding loop with the conserved Ile220 and Gly222 residues is situated (Figure 7B). The location of the mutation thus may help in tracing the signal transmission route along the EF-Tu–aminoacyl-tRNA interface. In the G222D mutant, the propensity of EF-Tu to follow the codon-driven conformational change of the aminoacyl-tRNA may be limited at low Mg^{2+} concentration due to an improper structural environment of the acceptor arm. This may be due to charge repulsion between the phosphates of the 3′ acceptor end and the negatively charged Asp222. Screening the latter charge by a divalent cation such as Mg^{2+} (Richardson and Richardson, 1990) may reduce the repulsion and thus restore the ability of both aminoacyl-tRNA and EF-Tu to reach the transient conformational state leading to GTP hydrolysis and the subsequent steps of A-site binding. Another factor contributing to the defective behavior of the mutant might be a perturbation of the interactions of the factor with ribosomal elements required for GTPase activation following the codon-induced structural change. Such potentially involved EF-Tu–ribosome interactions have been characterized in the 530 loop of 16S RNA (Powers and Noller, 1993; Noller *et al.*, 1996), in domain II of 23S rRNA (GTPase-associated region; Rosendahl and Douthwaite, 1994) and in domain VI of 23S rRNA (α-sarcin loop; Moazed *et al.*, 1988; Tapprich and Dahlberg, 1990).

Kirromycin is known to perturb the function of EF-Tu by freezing the protein in a GTP-like conformation with high affinity for the ribosomal A site (Parmeggiani and Swart, 1985; Mesters *et al.*, 1994). In time-resolved fluorescence experiments such as those performed in Figures 3 and 4, the high fluorescence intermediate complexes with EF-Tu were shown to be frozen with kirromycin, i.e. the antibiotic is freezing the factor in the GTPase conformation (Rodnina *et al.*, 1994, 1995). Thus, the absence of the high fluorescence intermediates in EF-Tu(G222D) complexes at the near physiological concentration of 5 mM Mg^{2+} (see Figures 3C and 4B) points to a defect in the high affinity interaction with the ribosome. This finally provides a good explanation for the recessive nature of the kirromycin sensitivity of EF-Tu(G222D), in contrast to the dominant sensitivity of wild-type EF-Tu, in cells that also contain kirromycin-resistant mutant EF-Tu (see Introduction). In future experiments, the remarkable synergism between EF-Tu(G222D) and the latter will be

analyzed in order to explain the role of the defective EF-Tu(G222D) in the observed phenotype of enhanced translational inaccuracy.

Materials and methods

Buffer and reagents

Buffer A: 25 mM Tris–HCl, pH 7.5, 50 mM NH_4Cl, 1 mM dithioerythritol, and the indicated concentration of $MgCl_2$; for instance, buffer A(5) contains 5 mM $MgCl_2$. Poly(U), poly(A) and other biochemicals were purchased from Boehringer-Mannheim. Radioactive compounds were from Amersham or ICN. The fluorescent GTP derivative, 3′-*O*-(*N*-methylanthranilyl)-2′-deoxyguanosine triphosphate (mant-dGTP), was donated by R.Goody.

tRNAs and proflavin-labeled tRNA derivatives

$tRNA^{Phe}$ from brewer's yeast (charging capacity of 1.7 nmol/A_{260} unit) was purchased from Boehringer-Mannheim. AcPhe-$tRNA^{Phe}$ was prepared and purified to homogeneity (1.75 nmol/A_{260} unit) by HPLC on a C-4 column (Rodnina and Wintermeyer, 1992). The fluorescent derivative $tRNA^{Phe}$(Prf16/17) was prepared as described previously (Wintermeyer and Zachau, 1979) and was chargeable to 1.5 nmol/A_{260} unit.

Ribosomes and elongation factor Tu

Tight-coupled 70S ribosomes from *E.coli* MRE 600 were prepared as described (Rodnina and Wintermeyer, 1995). On the basis of 23 pmol/A_{260} unit, the activity of the ribosomes was 85–90% in binding AcPhe-$tRNA^{Phe}$ to both A and P sites and 80–85% in peptide bond formation. Ribosome concentrations given in the text are based on tRNA binding activity. EF-Tu was prepared from *E.coli* K12 by the previously described procedure (Rodnina and Wintermeyer, 1995). For experiments with mant-dGTP, nucleotide-free EF-Tu was prepared as described (Rodnina and Wintermeyer, 1995). Wild-type EF-Tu and mutant EF-Tu(G222D), both with a C-terminal tag of $Ser(His)_6$, were overproduced in *E.coli* JM109 and purified as described (Boon *et al.*, 1992, 1995). After the final purification step by FPLC on Mono Q, the protein was concentrated by precipitation with 50% ammonium sulfate, redissolved and dialyzed against buffer A(7), supplemented with 10 μM GDP and 5% (v/v) glycerol, and stored at –80°C. Protein concentrations were determined according to Bradford (1976) using pyruvate kinase as a standard.

Preparation of EF-Tu·GTP·aminoacyl-tRNA complexes

About 3 nmol of EF-Tu·GDP were incubated for 15 min at 37°C with 1 mM GTP, 3 mM phosphoenolpyruvate and 20 μg/ml pyruvate kinase in 130 μl of buffer A(5). About 2 nmol of $tRNA^{Phe}$ or $tRNA^{Phe}$(Prf16/17) were aminoacylated with ^{14}C-labeled (1037 d.p.m./pmol) or ^{3}H-labeled (8000 d.p.m./pmol) phenylalanine in 100 μl of buffer A(5), as described (Rodnina and Wintermeyer, 1995). Subsequently, the two solutions were mixed and incubated for another 5 min at 37°C. The ternary complex was purified by gel filtration on Superdex 75 (Pharmacia) in buffer A(5) (Rodnina *et al.*, 1994). The complex eluted at a concentration of ~0.5–1.0 μM; it was stored on ice and diluted to the desired concentration immediately before use. Ternary complex containing mant-dGTP was prepared in the same way, except that nucleotide-free EF-Tu was used for complex formation.

Preparation of ribosome complexes

To prepare ribosome complexes with an occupied P site, the ribosomes were incubated with a 1.1-fold excess of AcPhe-$tRNA^{Phe}$ and 1 mg/ml of poly(U) in buffer A(10) for 15 min at 37°C. Alternatively, 0.2 mg/ml of poly(A) was used.

Kinetic experiments

Fluorescence stopped-flow measurements were performed and the data evaluated as described previously (Rodnina *et al.*, 1996). The fluorescence of proflavin was excited at 436 nm and measured with two photomultipliers after passing KV 500 filters (Schott); mant-dGTP fluorescence was excited at 363 nm and measured after passing KV 408 filters (Schott). The data were evaluated by fitting an expression which contained the sum of up to two exponential terms (with characteristic time constants, k_{app} and amplitudes, A) and another variable for the final signal. With the apparatus used, time constants of up to 500/s could be measured.

The experiments were performed at 20°C by rapidly mixing equal volumes (60 μl each) of the respective ternary complex, purified by gel filtration, and the ribosome complex. The reproducibility of the rate constants was ~10%, that of the amplitudes was 15% (within one experiment) and the reproducibility from shot to shot was ~5% for both parameters.

Quench-flow experiments were performed using a KinTek quench-flow apparatus under the conditions of the stopped-flow experiments. To measure the rates of GTP hydrolysis, equal volumes (26 μl each) of ribosome complex and purified ternary complex containing [γ-^{32}P]GTP were mixed rapidly and, after the desired incubation time, the reaction was terminated by quenching with 1 M $HClO_4$/3 mM potassium phosphate. After centrifugation, an aliquot from the supernatant was extracted with isopropyl acetate in the presence of sodium molybdate (Parmeggiani and Sander, 1981), and the radioactivity in the non-aqueous supernatant counted in a liquid scintillation cocktail (QS501 with 30% Triton X-100). To measure the rate of dipeptide formation, ribosome complexes containing HPLC-purified, unlabeled AcPhe-$tRNA^{Phe}$ in the P site and EF-Tu complexes containing [^{3}H]Phe-$tRNA^{Phe}$ (8000 d.p.m./pmol) were used. The reaction was stopped with 0.5 M KOH, incubated for 30 min at 37°C, neutralized and peptides were analyzed by HPLC (Rodnina and Wintermeyer, 1995).

To determine individual rate constants, ribosome titrations were performed, i.e. apparent first-order rate constants were measured at a fixed concentration of EF-Tu·GTP·Phe-$tRNA^{Phe}$ (0.1 μM) and increasing concentrations of ribosomal complex (from 0.3 up to 2.5 μM). For the poly(A) system, rate constants were determined from the concentration dependence of the apparent rate constants on the basis of a two-step model (Bernasconi, 1976):

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} D$$

For states C and D, the relative fluorescence quantum yields were calculated from the time course of the reaction at 20°C and 10 mM Mg^{2+}, setting the initial fluorescence of the ternary complex to 1, and taking into account the rate constants k_1, k_{-1}, k_2 and k_{-2} determined from the titration. The fitting was performed as described (Rodnina *et al.*, 1996), assuming pseudo-first-order conditions with respect to the ribosome concentration.

The dependence of the fluorescence of A (free ternary complex) and of D (ribosome-bound ternary complex) on Mg^{2+} concentration was measured at a saturating concentration of poly(A)-programed ribosomes. The fluorescence of Phe-$tRNA^{Phe}$(Prf16/17) both in the free ternary complex and in the ribosome-bound state was not affected by Mg^{2+} (3.5–10 mM Mg^{2+}, data not shown); thus, the relative fluorescence quantum yields of states A, C and D, measured at 10 mM Mg^{2+}, were used for fitting the time courses of the initial binding at lower Mg^{2+} concentrations for the determination of the individual rate constants of the reaction (Rodnina *et al.*, 1996).

For the poly(U)-system, the following model was adopted:

$$\text{Steps}\quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7$$
$$A + B \rightleftharpoons C \rightleftharpoons D \rightleftharpoons E \longrightarrow F \longrightarrow G \longrightarrow H \longrightarrow I$$

Step 1 represents the initial interaction of the ternary complex with the ribosome, reflected in a fluorescence increase of proflavin in the D loop of $tRNA^{Phe}$ in the ternary complex (Rodnina *et al.*, 1996). Step 2 reflects codon–anticodon recognition, observed as an increase of the fluorescence of wybutine in the anticodon loop of $tRNA^{Phe}$ or as a parallel change of the fluorescence of proflavin in the tRNA (Rodnina *et al.*, 1994); the latter effect is used in the present study to follow the kinetics of codon–anticodon interaction. For the calculations, the rate constant of the back reaction of step 2 was considered to be negligibly small (Karim and Thompson, 1986). Codon–anticodon recognition is transmitted to EF-Tu leading to a kinetically coupled conformational change represented by step 3 which is reported by mant-dGTP fluorescence. The rate of GTP hydrolysis (step 4) was measured by the quench-flow technique. The following steps 5, 6 and 7 represent the accommodation of aminoacyl-tRNA in the A site, dissociation of EF-Tu·GDP and peptide bond formation, respectively (Rodnina *et al.*, 1995). The latter step was also measured by quench-flow.

Fluorescence titrations

Fluorescence measurements were made on a Schoeffel RRS 1000 spectrofluorimeter. The excitation wavelength was 460 nm; proflavin emission was measured at 510 nm.

To determine the dissociation constant, K_d, of the initial binding complex, the fluorescence of 0.1–0.2 μM of purified EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe}(Prf16/17) was measured upon addition of increasing amounts (up to 2.5 μM) of poly(A)-programed ribosomes. The resulting fluorescence was corrected for dilution (<15%) and for background fluorescence (~2%). The data were evaluated by fitting the following equation:

$$y = \frac{x}{x + K_d}$$

where

$$y = \frac{F(c) - F_0}{F_{max} - F_0}$$

$$x = c_a - y \cdot c_t$$

F_0, initial fluorescence of the ternary complex; F_{max}, maximum fluorescence after addition of saturating amounts of ribosomes; $F(c)$, fluorescence at a given concentration of free ribosomes, x; c_a and c_t, added concentrations of ribosomes and ternary complex, respectively. The fitting program was TableCurve (Jandel). The number of Mg^{2+} ions participating in the interaction was estimated as described (Alberty, 1969).

Acknowledgements

We thank Vladimir Katunin for the preparation of AcPhe-tRNA^{Phe}, J.Martien de Graaf for the isolation of EF-Tu(G222D) and Roger Goody for mant-dGTP. This project has been supported by the Deutsche Forschungsgemeinschaft (Wi 626/11-1) and by the Netherlands Foundation for Chemical Research (SON 328-035).

References

Abrahams,J.P., Acampo,J.J.C., Ott,G., Sprinzl,M., de Graaf,J.M., Talens,A. and Kraal,B. (1990) The interaction between aminoacyl-tRNA and the mutant elongation factors Tu A_R and B_0. *Biochim. Biophys. Acta*, **1050**, 226–229.

Alberty,R.A. (1969) Standard free energy, enthalpy, and entropy changes as a function of pH and pMg for several reactions involving adenosine phosphates. *J. Biol. Chem.*, **244**, 3290–3302.

Berchtold,H., Reshetnikova,L., Reiser,C.O.A., Schirmer,K., Sprinzl,M. and Hilgenfeld,R. (1993) Crystal structure of active elongation factor Tu reveals major domain rearrangements. *Nature*, **365**, 126–132.

Bernasconi,C.F. (1976) *Relaxation Kinetics*. Academic Press, New York.

Boon,K., Vijgenboom,E., Madsen,L.V., Talens,A., Kraal,B. and Bosch,L. (1992) Isolation and functional analysis of histidine-tagged elongation factor Tu. *Eur. J. Biochem.*, **210**, 177–183.

Boon,K., Krab,I., Parmeggiani,A., Bosch,L. and Kraal,B. (1995) Substitution of Arg230 and Arg233 in *Escherichia coli* elongation factor Tu strongly enhances its pulvomycin resistance. *Eur. J. Biochem.*, **227**, 816–822.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, **72**, 248–254.

Clark,B.F.C., Kjeldgaard,M., La Cour,T.F.M., Thirup,S. and Nyborg,J. (1990) Structural determination of the functional sites of *E.coli* elongation factor Tu. *Biochim. Biophys. Acta*, **1050**, 203–208.

Duisterwinkel,F.J., de Graaf,J.M., Schretlen,P.J.M., Kraal,B. and Bosch,L. (1981) A mutant elongation factor Tu which does not immobilize the ribosome upon binding of kirromycin. *Eur. J. Biochem.*, **117**, 7–12.

Duisterwinkel,F.J. *et al.* (1984) Specific alterations of the EF-Tu polypeptide chain considered in the light of its three-dimensional structure. *EMBO J.*, **3**, 113–120.

Karim,A.M. and Thompson,R.C. (1986) Guanosine 5′-*O*-(3-thiotriphosphate) as an analog of GTP in protein biosynthesis. The effects of temperature and polycations on the accuracy of initial recognition of aminoacyl-tRNA ternary complexes by ribosomes. *J. Biol. Chem.*, **261**, 3238–3243.

Kjeldgaard,M., Nissen,P., Thirup,S. and Nyborg,J. (1993) The crystal structure of elongation factor EF-Tu from *Thermus aquaticus* in the GTP conformation. *Structure*, **1**, 35–40.

Mesters,J.R., Zeef,L.A., Hilgenfeld,R., de Graaf,J.M., Kraal,B. and Bosch,L. (1994) The structural and functional basis for the kirromycin resistance of mutant EF-Tu species in *Escherichia coli*. *EMBO J.*, **13**, 4877–4885.

Moazed,D., Robertson,J.M. and Noller,H. (1988) Interaction of elongation factors EF-G and EF-Tu with a conserved loop in 23S rRNA. *Nature*, **334**, 362–354.

Nissen,P., Kjeldgaard,M., Thirup,S., Polekhina,G., Reshetnikova,L., Clark,B.F.C. and Nyborg,J. (1995) Crystal structure of the ternary complex of Phe-tRNA, ET-Tu and a GTP analog. *Science*, **270**, 1464–1472.

Noller,H.F., Powers,T., Allen,P.N., Moazed,D. and Stern,S. (1996) rRNA and translation: tRNA selection and movement in the ribosome. In Zimmermann,R.A. and Dahlberg,A.E. (eds), *Ribosomal RNA: Structure, Evolution, Processing, and Function in Protein Biosynthesis*. CRC Press, Boca Raton, FL, pp. 239–258.

Pai,E.F., Krengel,U., Petsko,G.A., Goody,R.S., Kabsch,W. and Wittinghofer,A. (1990) Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 Å resolution: implications for the mechanism of GTP hydrolysis. *EMBO J.*, **8**, 2351–2359.

Parmeggiani,A. and Sander,G. (1981) Properties and regulation of the GTPase activities of elongation factors Tu and G, and of initiation factor 2. *Mol. Cell. Biochem.*, **35**, 129–158.

Parmeggiani,A. and Swart,G.W.M. (1985) Mechanism of action of kirromycin-like antibiotics. *Annu. Rev. Microbiol.*, **39**, 557–577.

Peter,M.E., Schirmer,N.K., Reiser,C.O.A. and Sprinzl,M. (1990) Mapping of the effector region in *Thermus thermophilus* elongation factor Tu. *Biochemistry*, **29**, 2876–2884.

Powers,T. and Noller,H.F. (1993) Evidence for functional interaction between elongation factor Tu and 16S RNA. *Proc. Natl Acad. Sci. USA*, **90**, 1364–1368.

Richardson,J.S. and Richardson,D.C. (1990) Principles and patterns of protein conformation. In Fasmann,G.D. (ed.), *Prediction of Protein Structure and the Principles of Protein Conformation*. Plenum Press, New York, pp. 1–98.

Rodnina,M.V. and Wintermeyer,W. (1992) Two tRNA-binding sites in addition to A and P sites on eukaryotic ribosomes. *J. Mol. Biol.*, **228**, 450–459.

Rodnina,M.V. and Wintermeyer,W. (1995) GTP consumption of elongation factor Tu during translation of heteropolymeric mRNAs. *Proc. Natl Acad. Sci. USA*, **92**, 1945–1949.

Rodnina,M.V., Fricke,R. and Wintermeyer,W. (1994) Transient conformational states of aminoacyl-tRNA during ribosome binding catalyzed by elongation factor Tu. *Biochemistry*, **33**, 12267–12275.

Rodnina,M.V., Fricke,R., Kuhn,L. and Wintermeyer,W. (1995) Codon-dependent conformational change of elongation factor Tu preceding GTP hydrolysis on the ribosome. *EMBO J.*, **14**, 2613–2619.

Rodnina,M.V., Pape,T., Fricke,R., Kuhn,L. and Wintermeyer,W. (1996) Initial binding of the elongation factor Tu·GTP·aminoacyl-tRNA complex preceding codon recognition on the ribosome. *J. Biol. Chem.*, **271**, 646–652.

Rosendahl,G. and Douthwaite,S. (1994) The antibiotics micrococcin and thiostrepton interact directly with 23S rRNA nucleotides 1067A and 1095A. *Nucleic Acids Res.*, **22**, 357–363.

Swart,G.W.M., Parmeggiani,A., Kraal,B. and Bosch,L. (1987) Effect of the mutation glycine-222→ aspartic acid on the functions of elongation factor Tu. *Biochemistry*, **26**, 2047–2054.

Tapprich,W.E. and Dahlberg,A.E. (1990) A single base substitution at position 2661 in *E.coli* 23S ribosomal RNA affects the binding of ternary complex to the ribosome. *EMBO J.*, **9**, 2649–2655.

Tubulekas,I. and Hughes,D. (1993) A single amino acid substitution in elongation factor Tu disrupts the interaction between ternary complex and the ribosome. *J. Bacteriol.*, **175**, 240–250.

Van der Meide,P.H., Duisterwinkel,F.J., de Graaf,J.M., Kraal,B., Bosch,L., Douglass,J. and Blumenthal,T. (1981) Molecular properties of two mutant species of the elongation factor Tu. *Eur. J. Biochem.*, **117**, 1–6.

Van Noort,J.M., Kraal,B., Sinjorgo,K.M.C., Persoon,N.L.M., Johanns, E.S.D. and Bosch,L. (1986) Methylation *in vivo* of elongation factor EF-Tu at lysine-56 decreases the rate of tRNA-dependent GTP hydrolysis. *Eur. J. Biochem.*, **160**, 557–561.

Vijgenboom,E. and Bosch,L. (1989) Translational frameshifts induced by mutant species of the polypeptide chain elongation factor Tu of *Escherichia coli*. *J. Biol. Chem.*, **264**, 13012–13017.

Vijgenboom,E., Vink,T., Kraal,B. and Bosch,L. (1985) Mutants of the elongation factor EF-Tu, a new class of nonsense supressors. *EMBO J.*, **4**, 1049–1052.

Wintermeyer,W. and Zachau,H.G. (1979) Fluorescent derivatives of yeast tRNA^{Phe}. *Eur. J. Biochem.*, **98**, 465–475.

Received on July 17, 1996; revised on August 26, 1996